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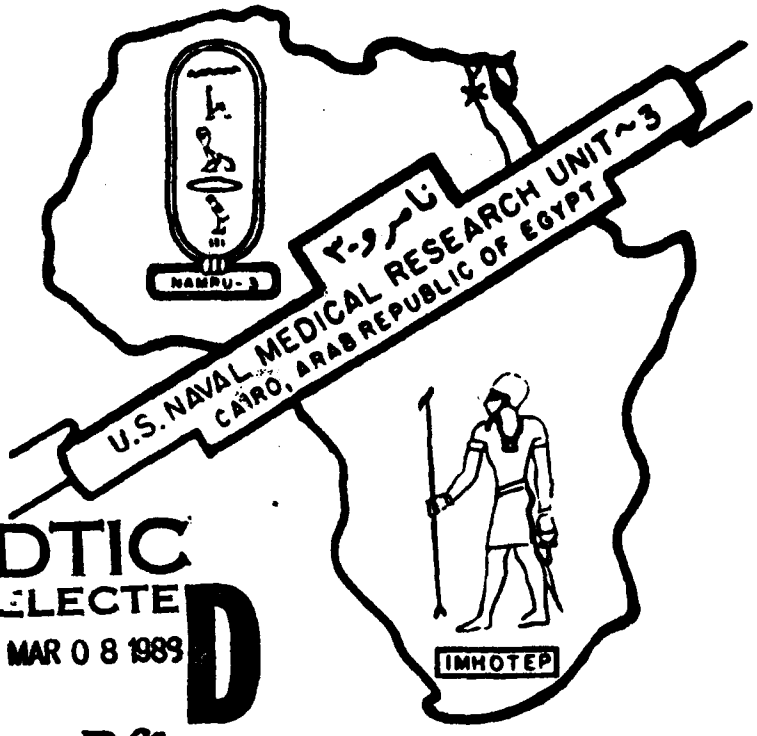

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Neuropeptide Modulation of Murine Erythropoiesis*†

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ABSTRACT

Neuropeptide modulation of hematopoietic stem cell differentiation was studied in an *in vitro* murine system. Endorphins were able to influence the erythropoietin-dependent differentiation of bone marrow cells into erythroid colony forming units in a dose dependent manner. The effects on progenitor cell maturation were influenced by the conditions and time of exposure to the endorphins. The modulation of erythropoiesis by the endorphins suggests that these peptides may function as modifiers of the maturation of bone marrow cells.

Introduction

Neuropeptides, particularly the endogenous opioid peptides, function in the central nervous system (CNS) as potent analgesics in response to pain stimuli.[3,4,8] One group of the endogenous opioid peptides, termed endorphins, is derived from beta-lipotropin, which, along with adrenocorticotropic hormone (ACTH), is a product of pro-opiomelanocorticotropin.[10] In addition, the enkephalins, synthesized in both the brain and the intestinal tract, are molecules with structural homology to the N-terminal pentapeptide of the endorphins.

Neuropeptides also modulate immunological functions; this supports the concept of regulatory links between the neuroendocrine and immune systems.[1,19] Most notably, the endorphins and the enkephalins modulate mitogenic

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responses of rodent and human lymphocytes,[5,12,17] increase the number of T-rosette cells,[15,16,21] influence specific antibody responses,[7] enhance natural killer cell activity,[11,17] and induce *in vitro* and *in vivo* chemotaxis in monocytes and neutrophils.[20] Furthermore, the presence of neuropeptides and receptors for these molecules has been detected in macrophages and on lymphocytes, respectively.[6,9] Hormone-like activities similar to those of endorphins have also been described after stimulation of the immune system.[19] In particular, the structural and biological activity of alpha-interferon is associated with endorphin-like and ACTH activities.[18]

The concept that hormones or other stimulus-derived molecules may have important primary or secondary influences on the immune and central nervous systems prompted this investigation of the role of neuropeptides on the differentiation of hematopoietic stem cells. In the present study, the neuropeptide influences on colony forming units-erythroid (CFU-e) are examined using murine erythroid progenitor cells.

Materials and Methods

Cells

Bone marrow cells from B6.C-*H*-2^{bm12} mice were used. These mice, which have a point mutation in the *I-A* region, are otherwise identical to C57BL/6J mice; the mutation in these mice does not affect erythroid differentiation of bone marrow cells.* The mice† were bred and housed in Thoren laminar flow, high efficiency particulate air-filtered caging systems with free access to food and water. All cages, bedding, food, and water were autoclaved prior to use.

Mice were killed by cervical dislocation and the bone marrow cells from the femurs and tibias were flushed using cold collecting medium: Iscoves' Modified Dulbecco's Minimal Essential Medium (IMDM), supplemented with 0.05 percent sodium bicarbonate, one mM sodium pyruvate, 0.1 mM nonessential amino acids, two mM L-glutamine, five I.U. per ml penicillin, five μg per ml streptomycin‡ and two percent fetal bovine serum.§

CFU-E Assay

The differentiation of progenitor cells into erythroid cells was measured in plasma clot cultures as previously described.[13,14] Neuropeptides were diluted in national collection of type cultures (NCTC) 109 medium‡ and added in volumes of 0.1 ml to a final 1.0 ml volume, containing 5×10^5 bone marrow cells, thrombin (one U per ml),‖ bovine serum albumin and L-asparagine,** erythropoietin,†† NCTC 109 medium, 10 percent fasting blood sugar (FBS)‡‡ and bovine citrated plasma.§§ Cells (5×10^4) in 0.1 ml were cultured in polyvinyl tissue culture wells‖‖ in a 37°, five percent CO_2 humidified atmosphere. Two days later, quadruplicate cultures were harvested, the clots were fixed with glutaraldehyde and stained with benzidine and hematoxylin. Colonies were identified by a minimum of eight benzidine-positive nucleated cells. Results are expressed as the mean number of CFU-e per 10^5 cells ± standard deviation (S.D.) of the assay or standard error (S.E.) when multiple experiments were per-

* Unpublished observations.

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formed. Significance was determined at the 95 percent confidence limit using the student's t-test.

Neuropeptides

Neuropeptides were used in these studies* and were stored at −70°C after dilution to one mg per ml in NCTC 109 medium.

Results

Effects of Pro-Opiomelanocorticotropic Hormone Derivatives on Erythropoiesis

Products of pro-opiomelanocorticotropic hormone were tested for their ability to modulate murine EP-dependent erythropoiesis. These assays were performed using a suboptimal concentration of erythropoietin (EP) such that any change in the numbers of CFU-e would be observed in the log phase of the dose response and not in the plateau phase of the response. Dose responses were included in each experiment to verify erythropoietin stimulation of CFU-e activity. Amounts of neuropeptides chosen were physiological.[10,21]

The results of representative experiments with selected concentrations of agents are summarized in table I. The data indicate that appropriate concentrations of products of beta-lipotropin, the enkephalins, and the endorphins, but not adrenocorticotropic hormone (ACTH) or alpha-melanocyte stimulating hormone, enhance the number of EP-dependent CFU-e. None of the neuropeptides tested stimulated erythropoiesis in the absence of EP (data not shown).

TABLE I

Effect of Selected Derivatives of Pro-opiomelanocorticotropic Hormone on Murine Erythropoiesis

Neuropeptide	Conc.* (ng/ml)	CFU-e/5x10⁴ BMc ± S.D.† EP only	EP + Neuropeptide	p‡
ACTH	100	47 ± 13	35 ± 16	0.15
alpha-MSH	100	98 ± 30	76 ± 21	0.16
Met-Enkephalin	100	171 ± 35	301 ± 7	0.002
Leu-Enkephalin	100	109 ± 14	238 ± 80	0.020
beta-Endorphin	10	142 ± 48	273 ± 90	0.020
Dynorphin	1	103 ± 16	200 ± 45	0.004

*Several concentrations of neuropeptides were tested in different experiments; the results are representative and include the erythropoietin control level of stimulation.

†Colony-forming units-erythroid per 5x10⁴ bone marrow cells per clot. Results are the mean ± S.D. of quadruplicate cultures in the presence of erythropoietin only (0.4 U/ml) or erythropoietin and neuropeptide cocultured for two days.

‡p value determined by paired student's t-test.

ACTH = adrenocorticotropic hormone
CFU-e = colony forming units - erythroid
EP = erythropoietin
BMc = bone marrow cells
MSH = melanocyte stimulating hormone

Stimulation of CFU-E by Endorphins

Experiments were performed to determine stimulatory concentrations and to compare the ability of the alpha- and beta-endorphins to enhance the EP-dependent stimulation of CFU-e differentiation (tables II and III). Differences in erythropoietin concentrations also influenced the effect of the neuropeptides. Low concentrations of alpha-endorphin were stimulatory when bone marrow CFU-e activity was low; when higher levels of CFU-e resulted, no effects of alpha-endorphin were observed. Beta-endorphin was inhibitory at high levels of CFU-e activity.

Effects of Endorphins on the Number of Erythroid Progenitor Cells

The data show that endorphins are capable of modulating the number of CFU-e in an EP- and dose-dependent manner. The effect of the endorphins is

* Accurate Scientific, Inc., Westbury, NY.

TABLE II

Effect of alpha-Endorphin on Murine Erythropoiesis

Group*	Dose a-Endorphin	CFU-e/10^5 cells ± S.E.†	Mean Percent Control‡
I	0	158 ± 22	100
	1 ng/ml	388 ± 79§	178
	10 ng/ml	218 ± 48§	121
	100 ng/ml	184 ± 74	176
	1000 ng/ml	243 ± 64	185
II	0	604 ± 56	100
	1 ng/ml	681 ± 59	113
	10 ng/ml	538 ± 55	91
	100 ng/ml	456 ± 41	76
	1000 ng/ml	495 ± 138	83

*Experiments in which low responses (Group I, n = 3) or high responses (Group II, n = 4) to erythropoietin occurred.
†Mean number of colony-forming units-erythroid from different experiments.
‡Mean percent change based on individual experiment values.
§Significant difference between stimulated and control mean ($p < 0.05$).
CFU-e = colony forming units - erythroid
S.E. = standard error

dependent on the presence of EP since no endogenous erythropoiesis was seen in the presence of neuropeptide and in the absence of EP (data not shown). To determine whether endorphins stimulated the differentiation of new progenitor cells capable of differentiation into CFU-e or enhanced the hormonal activity of EP, selected concentrations of alpha-endorphin were cultured with different concentrations of EP. The results of these studies are listed in table IV and indicate that the concentration of alpha-endorphin used for incubation did not increase the number of CFU-e above the number that were stimulated by an optimal concentration of EP alone.

Effects of Pre-Incubation of Bone Marrow Cells with Endorphins

To determine whether or not endorphins were required continuously, bone marrow cells were incubated with alpha-endorphin prior to culture with EP. The incubations with alpha-endorphin were done at either 4°C or 37°C to ascertain whether only binding of the alpha-endorphin to the cells was sufficient or whether metabolic events were also necessary for the subsequent effects on CFU-e differentiation. The cells were washed after incubation and before culture with EP. As shown in figure 1, the incubation of bone marrow cells at either 4°C or 37°C resulted in enhancement or depression, respectively, of the number of CFU-e after subsequent stimulation by EP. An EP dose response (not shown) was performed in each treatment group. In the comparison group, with no preincubation and with both EP and 0.01 μg per ml endorphin present during the plasma clot culture, the numbers of CFU-e were increased compared to the EP control, as expected. In contrast, the incubation of bone marrow cells with 0.01 μg per ml endorphin for two hours at either 4°C or 37°C resulted in a significant decrease in the number of CFU-e induced by EP ($p < 0.05$) compared to the response of cells cultured in medium

TABLE III

Effects of beta-Endorphin on Murine Erythropoiesis

Group*	Dose b-Endorphin	CFU-e 10^5 cells ± S.E.†	Mean Percent Control‡
I	0	224 ± 38	100
	1 ng/ml	384 ± 80	171
	10 ng/ml	412 ± 81§	184
	100 ng/ml	451 ± 64§	202
	1000 ng/ml	408 ± 76	213
II	0 ng/ml	652 ± 56	100
	1 ng/ml	564 ± 7	87
	10 ng/ml	550 ± 67	84
	100 ng/ml	417 ± 17§	64
	1000 ng/ml	448 ± 212	69

*Experiments in which low response (Group I, n = 4) or high response (Group II, n = 3) to erythropoietin occurred.
†Mean number of colony-forming units-erythroid from different experiments.
‡Mean percent change based on individual experiments.
§Significant difference between stimulated and control means ($p < 0.05$).
CFU-e = colony forming units - erythroid
S.E. = standard error

TABLE IV

Effect of Different Concentrations of Erythropoietin on Modulation of Erythropoiesis by alpha-Endorphin

EP (U/ml)	α-End (10 ng/ml)	CFU-e/10^5 Cells*	Mean Percent Control†
0.2	-	146	
	+	256‡	175
0.4	-	299	
	+	356	119
0.8	-	378	
	+	214	57
1.6	-	218	
	+	311	143

*Mean number of colony forming units-erythroid from quadruplicate cultures. The standard deviation of the cultures was less than 20 percent.
†Mean percent of control.
‡Indicates significant difference between mean values ($p < 0.05$).
EP = erythropoietin
α-End = alpha-Endorphin
CFU-e = colony forming units-erythroid

only. The results indicate that the addition of endorphin to bone marrow cells prior to plasma clot culture was sufficient to effect a decrease in the number of CFU-e; these responses were different from the responses of cells cultured simultaneously with both EP and endorphin; the absolute decrease in number of CFU-e of cells cultured at 37°C may be due to the loss of selective cell types by adherence or culture conditions.

Discussion

Neuropeptide modulation of murine hematopoiesis was demonstrated in an *in vitro* system measuring the differentiation of bone marrow progenitor cells into erythroid cells in the presence of erythropoietin. Results indicate that the hematopoietic system is modulated by neuroendocrine stimuli.

Endorphins are derived from beta-lipotropin, a product of pro-opiomelanocorticotropin. The endorphins are structurally homologous to several other neuropeptides, including dynorphins and enkephalins, as well as macrophage/fibroblast-derived alpha-interferon.[9,21] Because of this structural homology, it can be predicted that these molecules, although resulting from different types of stimuli, may indeed share biological activities in their respective environments. The structural similarity of these molecules may also be associated with a similar mode of biochemical or molecular action. Structural homology and biochemical action may also relate to a combined multisystem response to sensory or antigenically-foreign stimuli. Thus, in the immune system, the endorphins have multiple effects on the enhancement of antigen-specific responses of lymphocytes, macrophages/monocytes, and polymorphonuclear leuko-

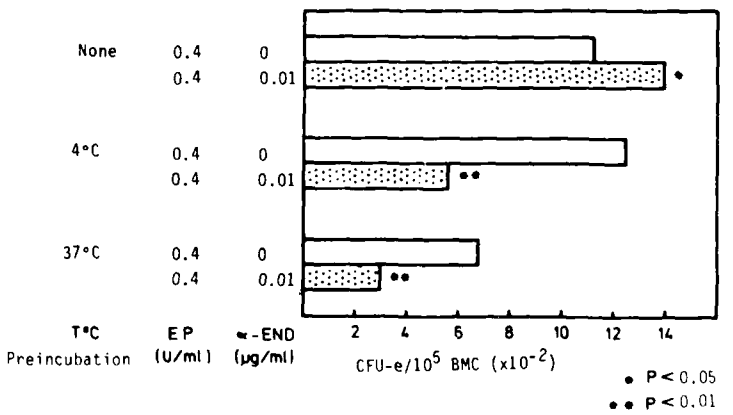

FIGURE 1 Effect of *in vitro* preincubation of mouse bone marrow cells with α-endorphin on subsequent erythropoietin-dependent stimulation of CFU-e formation.

cytes.[5,7,11,12,15,16,17,20,22] Similarly, alpha-interferon, a product of virus-induced stimulation, has biological activities of beta-lipotropin when tested in appropriate nervous system assays. Conversely, beta-lipotropin possesses interferon-like activities.[9] The results described in this report demonstrate that the endorphins and enkephalins are also able to influence hematopoietic, hormone-dependent differentiation of erythroid progenitor cells.

Neuropeptide effects on the modulation of erythropoietin-dependent differentiation of progenitor cells are not a substitution for the hormonal stimulation by erythropoietin but a modifier of the processes initiated by the hormone. None of the doses of neuropeptides tested stimulated the formation of CFU-e in the absence of erythropoietin, suggesting that differentiation was not mediated through a structural homology between neuropeptides and erythropoietin hormone molecules.

The mode of action of endorphins with regard to CFU-e differentiation is unclear. Experiments to assess whether or not endorphins are required to be present for the duration of culture indicated that modulatory effects are seen under conditions of both preincubation and co-culture. The effects differ, however, depending upon the nature of the incubation condition. In contrast to modulation in co-culture, which results in the enhancement of the number of CFU-e formed, a decrease in the number of CFU-e generated is seen after a two-hour preincubation period, regardless of the temperature. Preliminary experiments incubating erythropoietin and alpha-endorphin together have shown no interaction between these molecules resulting in either enhancement or inhibition.

Modulatory events of endorphins may occur at the level of different cell types through the following possible mechanisms: (1) increase the sensitivity of receptors for erythropoietin such that the response of bone marrow cells to endorphin at low concentrations of erythropoietin results in an optimal response; (2) stabilization of the erythropoietin hormone structure such that maximum biochemical and biologic activity is possible; (3) endorphin-mediated stimulation of an accessory cell resulting in the secretion of cellular factors which promote erythropoietin-induced cell differentiation; or (4) endorphin-mediated inhibition of factors which block progenitor cell differentiation.

The number of CFU-e generated in the presence of neuropeptides does not increase the total number of erythroid progenitor cells capable of maturation. These data indicate that endorphin-specific modulation of bone marrow cells results in both enhancement and suppression, depending on the dose and conditions of treatment. The decreased response, resulting from preincubation or from concomitant incubation with erythropoietin and bone marrow cells, may also reflect changes in the density of receptors for erythropoietin such that different concentrations of erythropoietin may be required. Preincubation at 4°C is effective, indicating that binding, and not processing of the endorphin signal, is sufficient as an initial step for the subsequent modulation of differentiation processes.

In summary, these results document a substantial and significant modulation by neuropeptides, particularly the endorphins, on the differentiation of bone marrow cells into erythroid progenitors, in a murine *in vitro* system. Interactions, therefore, exist between hormones of the central nervous and the hematopoietic systems.

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